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Positional and additive effects of basic amino acids on processing of precursor proteins within the constitutive secretory pathway

Toshio Watanabe[a], Kazuo Murakami[a] and Kazuhisa Nakayama[b]

[a]*Institute of Applied Biochemistry* and [b]*Institute of Biological Sciences and Gene Experiment Center, University of Tsukuba, Tsukuba, Ibaraki 305, Japan*

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We have recently shown that the Arg/Lys-X-Lys/Arg-Arg or Arg/Lys-X-X-X-Lys/Arg-Arg sequence serves as a signal for cleavage of precursor proteins within the constitutive secretory pathway, and this cleavage is catalyzed by furin, a mammalian homolog of the yeast Kex2 protease. In this study, we further examined sequence requirements for the constitutive precursor cleavage. Based on the data concerning cleavage efficiencies of various prorenin mutants with amino acid substitution(s) around the native cleavage site expressed in CHO cells, we revised the sequence rules that govern the constitutive cleavage as follows: (i) the Arg residue at position −1 is essential; (ii) in addition to the Arg at position −1, at least two out of the three basic residues at positions −2, −4, and −6 are required for efficient cleavage (the presence of all the three basic residues results in most efficient cleavage); (iii) at position +1, a hydrophobic aliphatic amino acid is not suitable.

Basic amino acid; Furin; Pro-protein cleavage; Prorenin; CHO cell

1. INTRODUCTION

Limited endoproteolysis of larger precursors is one of the key steps to generate bioactive peptides and proteins. In endocrine cells, peptides are produced from precursors through cleavage at pairs of basic amino acids (Lys–Arg or Arg–Arg) within the regulated secretory pathway [1]. Recently, PC2 [2,3] and PC1/3 [2–4], both of which are mammalian homologs of the yeast processing protease, Kex2 [5], have been shown to be involved in the regulated precursor cleavage at dibasic sites. On the other hand, in non-endocrine cells many secretory and membrane proteins have been thought to be produced from precursors through cleavage at multiple, rather than paired, basic sites within the constitutive secretory pathway. The regulated pathway is specifically present in endocrine cells, and serves to process precursors to mature peptides and to store the peptides in secretory granules until their release is stimulated, while the constitutive pathway is present in all types of cells, and serves to release molecules continuously without storage [6].

We have recently noticed that most of the constitutively processed precursors possess the consensus Arg-X-Lys/Arg-Arg (RXK/RR) motif at the cleavage sites [7]. Based on the data regarding cleavage efficiencies of prorenin mutants with amino acid substitution(s) around the cleavage site expressed in CHO cells, we have presented the following sequence rules that govern the constitutive precursor cleavage [7,8]. (i) A basic residue at the 4th (position −4) or 6th (position −6) residue upstream of the cleavage site besides the basic residues at positions −2 and −1 is required. (ii) At position −2, Lys is more favorable than Arg. (iii) At position −4, Arg is more favorable than Lys. (iv) At position +1, a hydrophobic aliphatic residue is not suitable. By expression studies in cultured cells [7,9] as well as in vitro experiments using the pure protease and substrates [9,10], we have shown that the cleavage of precursors with the sequence fitting the above rules is catalyzed by furin, which is another mammalian Kex2-like endoprotease [6] expressed in all tissues and cell lines [11]. In this study, we further examined sequence requirements for the constitutive cleavage and revised the sequence rules.

Correspondence address: K. Nakayama, Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305, Japan.

2. MATERIALS AND METHODS

Site-directed mutagenesis to substitute amino acid(s) around the Lys–Arg cleavage site of mouse *Ren-2* prorenin was performed using an appropriate oligonucleotide primer as described previously [12]. The mutated cDNA was subcloned into the pSVD vector [13]. DNA transfection and identification of produced renin molecules were performed as described previously [7,8]. Briefly, cells at ~70% confluence on a 35-mm dish were transfected with the prorenin plasmid. After 48 h incubation, the cells were radiolabeled with 0.2 mCi/ml of EXPRE^{35}S^{35}S (Du Pont-NEN) for 10 h. The medium was then collected, immunoprecipitated with anti-*Ren-2* renin antiserum, and analyzed by SDS-PAGE followed by fluorography. The efficiency of prorenin cleavage was estimated by densitometric scanning of bands of prorenin and renin in the autoradiogram.

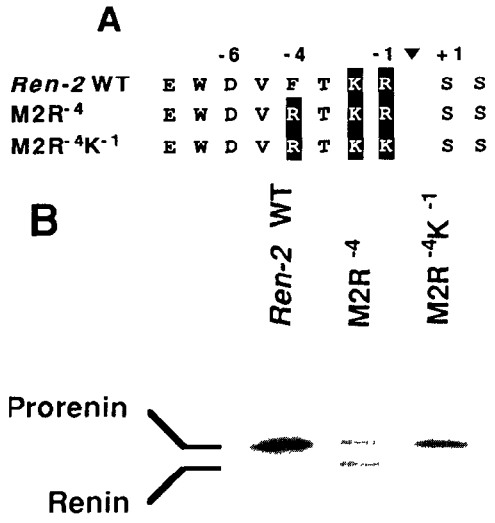

Fig. 1. Requirement of the Arg residue at position −1. (A) Amino acid sequences of the pertinent region of native and mutant mouse *Ren-2* prorenin. The cleavage site is indicated by an arrowhead, and basic residues are shown in dark boxes. (B) CHO cells transfected with the expression plasmid for prorenin shown in A were radiolabeled, and the secreted renin molecules were analyzed. Experimental details were described in section 2.

3. RESULTS AND DISCUSSION

We did not examined the effect of the Arg residue at position −1 in the earlier studies [7,8]. Therefore, we first addressed this issue. As shown in Fig. 1, the RXKR mutant ($M2R^{-4}$) of *Ren-2* prorenin was efficiently cleaved in CHO cells, while cleavage of the RXKK mutant ($M2R^{-4}K^{-1}$) was not detected. These results indicate that the Arg residue at position −1 is essential for constitutive precursor cleavage. This conclusion is supported by the fact that all the constitutively processed precursors have an Arg residue at position −1 [7]. Taken together with the data that PC1/3 [5] and Kex2 [14] also require the Arg at position −1, the requirement of Arg at position −1 may be a common feature of the Kex2 family of endoproteases.

We have previously shown that, in concert with a pair of basic residues at positions −1 and −2, a basic residue at position −4 or −6, but not at position −3 or −5, serves as a signal for the constitutive precursor cleavage [8]. To examine if these basic residues have an additive effect on the constitutive cleavage, we expressed the prorenin mutants shown in Fig. 2 in CHO cells. The cleavage efficiencies of the RRKR ($M2R^{-4}R^{-3}$) and RRXKR ($M2R^{-5}R^{-4}$) mutants (~50% each) were similar to that of the RXKR mutant ($M2R^{-4}$). By contrast, the RXRXKR mutant ($M2R^{-6}R^{-4}$) was cleaved almost completely (>95%). These results indicate that the basic residues at positions −4 and −6 have an additive effect on constitutive precursor cleavage.

The above data indicate that the presence of the basic residue at position −4 or −6 in addition to those at positions −1 and −2 is essential for constitutive cleavage, and the presence of those at all the four positions (−1, −2, −4, and −6) results in the most efficient cleavage. However, there is an alternative interpretation; in addition to the Arg at position −1, two out of the three basic residues at positions −2, −4, −6 are required, and the presence of those at all the three positions (−2, −4, and −6) results in the most efficient cleavage. If so, it is possible that the basic residue at position −2 may be dispensable if those at positions −4 and −6 are present. To examine this possibility, we expressed another prorenin mutant with an RXRXXR sequence ($M2R^{-6}R^{-4}Q^{-2}$) in CHO cells (Fig. 3). As expected, $M2R^{-6}R^{-4}Q^{-2}$ was cleaved, although with a slightly lower efficiency (~40%) than that of $M2R^{-4}$, while the RXXR ($M2R^{-4}Q^{-2}$) or RXXXXR ($M2R^{-6}Q^{-2}$) mutant was not cleaved. These results confirm the above speculation that, besides the Arg at position −1, at least two out of the three basic residues at positions −2, −4, and −6 are required for the constitutive cleavage.

By expression in CHO cells of various prorenin mutants with amino acid substitution(s) around the cleavage site, we have previously presented some sequence rules that govern constitutive precursor cleavage [9]. We extend here the previous study and revise the rules as follows. (i) The Arg at position −1 is essential. (ii) In

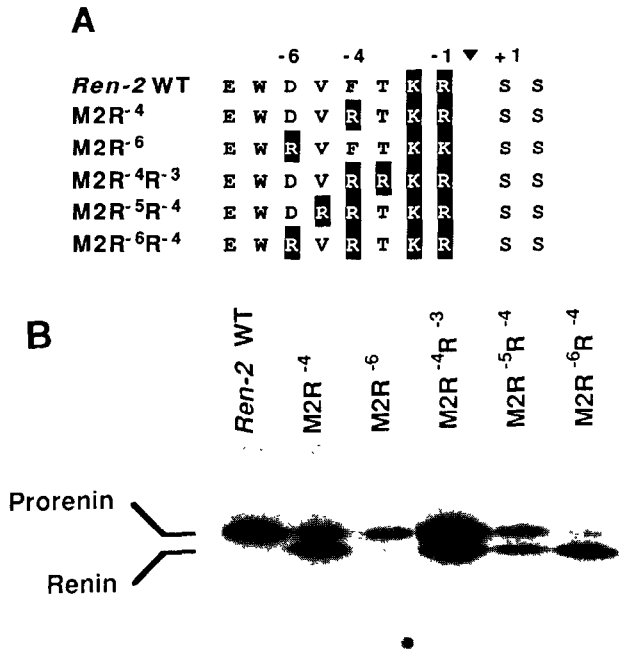

Fig. 2. Additive effect of the basic residues at positions −4 and −6. (A) Amino acid sequences of the pertinent region of native and mutant mouse *Ren-2* prorenin. The cleavage site is indicated by an arrowhead, and basic residues are shown in dark boxes. (B) CHO cells transfected with the expression plasmid for prorenin shown in A were radiolabeled, and the secreted renin molecules were analyzed. Experimental details were similar to those in Fig. 1.

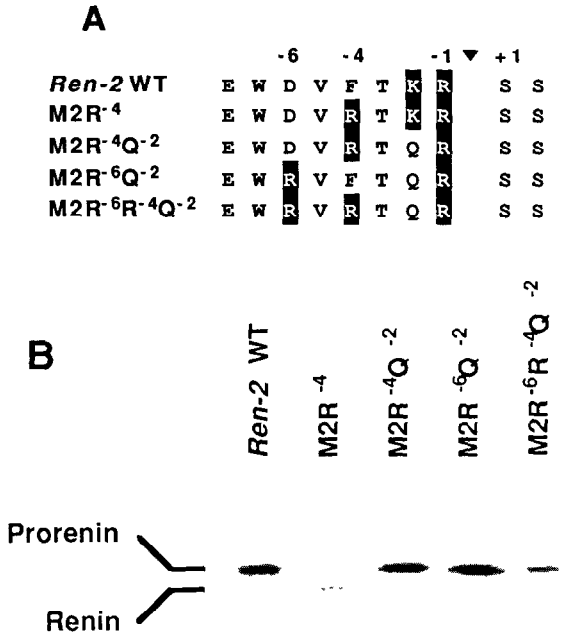

Fig. 3. Dispensability of the basic residue at position −2. (A) Amino acid sequences of the pertinent region of native and mutant mouse *Ren-2* prorenin. The cleavage site is indicated by an arrowhead, and basic residues are shown in dark boxes. (B) CHO cells transfected with the expression plasmid for prorenin shown in A were radiolabeled, and the secreted renin molecules were analyzed. Experimental details were similar to those in Fig. 1.

addition to the Arg at position −1, at least two out of the three basic residues at positions −2, −4, and −6 are required (the presence of all the three basic residues produces the most efficient cleavage). (iii) At position +1, a hydrophobic aliphatic residue is not suitable. The remarkable finding in this study is that the basic residue at position −2 is dispensable for constitutive cleavage if those at positions −4 and −6 are present; that is, the pair of basic residues at positions −2 and −1 are not essential. We have recently shown that the precursor cleavage within the regulated secretory pathway follows similar but not identical rules [15]. The difference between the constitutive and regulated cleavages is that the former and the latter require at least two and one, respectively, out of the three basic residues at positions −2, −4, and −6, besides the Arg at position −1.

Based on these data, we speculate that processing endoproteases possess some subsites containing negatively charged residues, which may interact with the positively charged residues of substrates (Fig. 4). The affinity of the substrates to the endoproteases may be determined by the number of interactions between the negative charges of the subsites and the positive charges of the substrates. This speculation is supported by the data of Van de Ven and colleagues [16,17]. By molecular modeling, they have pointed out the presence of some clusters of acidic residues on the substrate binding face in the catalytic domains of the Kex2 family of endoproteases, and have proposed that these acidic clusters may be involved in recognition of the basic residues of substrates.

The rules provide explanations for some unsolved problems as to the constitutive precursor cleavage. First, in the brain, human growth hormone-releasing hormone (hGRH) is produced as two forms, GRH(1–44)-NH2 and GRH(1–40), both of which are produced from pro-hGRH through cleavage at sites marked by the RXXR motif as a signal for precursor cleavage within the regulated secretory pathway [15]. Frohman et al. [18] have examined molecular forms of GRH in various tissues of transgenic mice bearing the hGRH gene linked to the metallothionein promoter. In neural and endocrine tissues, both GRH(1–44)-NH_2 and GRH(1–40) are produced, while only GRH(1–44)-NH_2 is detected in hepatic cells which possess only the constitutive pathway. Surveilance of the pro-hGRH sequence reveals that the cleavage site sequence to yield GRH(1–44)-NH_2 but not GRH(1–40) fits the rules presented here (Fig. 5). Second, Foster et al. [19] have reported that pro-protein C expressed in BHK cells is converted to the 2-chain mature form with an efficiency of ~30%. The cleavage site sequence contains basic residues at positions −1, −2, and −6 (Fig. 5), although the authors have not pointed out the importance of the Arg at position −6. Their important finding is that a pro-protein C mutant with another Arg at position −4 is converted completely to the 2-chain form (Fig. 5). This phenome-

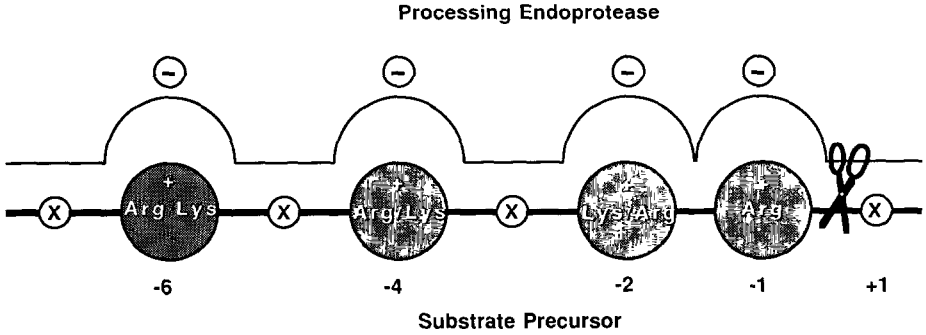

Fig. 4. A model for recognition of substrate precursor by processing endoprotease.

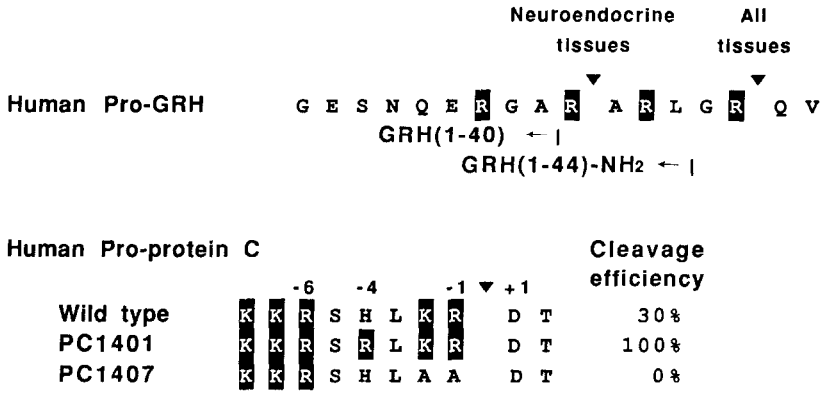

Fig. 5 The amino acid sequences of the pertinent regions of pro-hGRH, and human pro-protein C and its mutants. The cleavage sites are indicated by arrowheads, and basic residues are shown in dark boxes.

non could be explained by the additive effect of the Arg residues at positions −4 and −6.

In view of the presence of at least six Kex2 homologs in mammals (furin, PC2, and PC1/3 [5], and the most recently discovered PC4 [20], PACE4 [21], and PC6 [22]) the precursor cleavage is thought to be regulated in a very complex manner. As for the constitutive precursor cleavage, it is of great interest that the PACE4 transcript is detectable in all examined tissues and cell lines [21], like the furin transcript [11]. Therefore, both furin and PACE4 could be involved in constitutive cleavage. Comparative data on the cleavage selectivity of these endoproteases would improve future comprehension of the mechanism of the regulation of the precursor cleavage.

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